APPLICATION FOR UNITED STATES LETTERS PATENT

for

METHODS AND COMPOSITIONS RELATING TO IMPROVED
LENTIVIRAL VECTORS AND THEIR APPLICATIONS

by

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BACKGROUND OF THE INVENTION

The present application claims the benefit of U.S. Provisional Application Serial No. 60/248,398 filed on November 13, 2000, the entire text of which is herein incorporated by reference.

1. Field of the Invention

The present invention relates to improved lentiviral vectors and their use in gene delivery and high level expression of desired transgenes to target cells, particularly to human hematopoietic progenitor cells and differentiated blood lineages.

2. Description of Related Art

Gene therapy via the transduction of human hematopoietic stem cells (hHSC) represents a very promising approach for the treatment of a number of inherited and acquired lympho-hematological disorders. The stable genetic manipulation of long term repopulating hHSC with existing gene delivery systems, however, has been impossible to achieve at an efficiency compatible with therapeutic realities. Oncoretroviral vectors derived from Moloney murine leukemia virus (MLV), for instance, although highly appealing since they integrate their cargo into the chromosomes of target cells, cannot transduce hHSC that have not been first treated with inducers of proliferation (Kohn *et al.*, 1991; Mazurier *et al.*, 1998). Indeed, the nuclear transport of the MLV preintegration complex requires the breakdown of the nuclear envelope that occurs at mitosis (Roe *et al.*, 1993; Lewis and Emerman, 1994). Unfortunately hHSCs, whether harvested from the bone marrow (BM), the umbilical cord (UC) or mobilized in the peripheral circulation, are mostly quiescent and lose their pluripotentiality after stimulation and proliferation (Bhatia *et al.*, 1997; Dao *et al.*, 1997; Dorrell *et al.*, 2000). Recent reports, however, have shown that a significant fraction of pluripotent cells as well as cells capable of long-term engraftment in non-obese diabetic/severe combined immunodeficient (NOD/SCID), also called SCID-repopulating cells (SRC), can be maintained, transduced and even expanded using specific stimulation conditions (Dorrell *et al.*, 2000; Dao *et al.*, 1998; Piacibello *et al.*, 1999; Ueda *et al.*, 2000).

Lentiviruses are a subgroup of retroviruses that can infect nondividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Correspondingly, lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) can mediate the efficient delivery, integration and long-term expression of transgenes into non-mitotic cells both *in vitro* and *in vivo* (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b; Blomer *et al.*, 1997). In particular, HIV-based vectors can efficiently transduce human CD34$^+$ hematopoietic cells in the absence of cytokine stimulation (Akkina *et al.*, 1996; Sutton *et al.*, 1998; Uchida *et al.*, 1998; Miyoshi *et al.*, 1999; Case *et al.*, 1999), and these cells are capable of long-term engraftment in NOD/SCID mice (Miyoshi *et al.*, 1999). Furthermore, bone marrow from these primary recipients can repopulate secondary mice with transduced cells, confirming the lentivector-mediated genetic modification of very primitive hematopoietic precursors, most probably *bona fide* stem cells. Since none of the other currently available gene delivery systems has such an ability, lentiviral vectors provide a previously unexplored basis for the study of hematopoiesis and for the gene therapy of inherited and acquired lympho-hematopoietic disorders via the genetic modification of HSCs.

The demonstration of this important point, however, was provided with an early generation of lentiviral vectors unsuitable for therapeutic applications, either because they failed to meet biosafety requirements (Akkina *et al.*, 1996; Sutton *et al.*, 1998; Uchida *et al.*, 1998) or because they induced levels of transgene expression that were dismissingly low (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000). Accordingly, there is a significant need to develop improved lentiviruses for use as transducing vectors that are capable of effectively transducing hematopoietic cells, particularly hematopoietic progenitor cells, and which are capable of expressing desired transgenes at high levels.

SUMMARY OF THE INVENTION

The present invention is directed to the development of improved lentiviral vectors that both meet biosafety requirements, and which induce high levels of transgene

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expression. Accordingly, the present invention describes gene transfer vehicles that appear particularly well suited for the transduction of human hematopoietic precursor cells (HPCs) and for the expression of transgenes in differentiated blood lineages. These vectors will facilitate the further use of lentiviral vectors for the genetic manipulation of lympho-hematopoietic cells, and should be particularly useful for both research and therapeutic applications. However, it will be understood by the skilled artisan that the invention is not limited to the transduction of hematopoietic cells and that one may use the lentiviral vectors of the invention for the expression of transgenes in other cell types as well. Some examples of other cell types contemplated include terminally differentiated cells such as neurons, lung cells, muscle cells, liver cells, pancreatic cells, endothelial cells, cardiac cells, skin cells, bone marrow stromal cells, and eye cells. Additionally, stem cells and progenitor cells such as pancreatic ductal cells, neural precursors, and mesodermal stem cells are also contemplated.

The present invention thus concerns, in a general and overall sense, improved vectors that are designed to permit the transfection and transduction of human hematopoietic progenitor cells, or stem cells (hHSC), and provide high level expression of desired transgenes in such cells. The vectors of the present invention may be referred to as self-inactivating lentivectors due to the presence of lentiviral elements as well as certain "self-inactivating" design characteristics that render these vectors safe for human applications.

The lentivectors of the present invention provide, for the first time, an efficient means of achieving high level expression of desired trangenes in hHSCs, cells which have been difficult to transfect and transduce due to the fact that in their unstimulated state, they are relatively resistant to transduction by previous vector systems. These lentivectors have the ability to infect non-dividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Moreover, preferred lentiviral vectors of the present invention can mediate the efficient delivery, integration and long-term expression of transgenes into non-mitotic cells both *in vitro* and *in vivo*, even in the absence of cytokine stimulation. Stem cells

transduced by the more preferred lentivectors of the present invention are capable of long-term engraftment, for example, in NOD/SCID mice. Most notably, however, the more preferred lentivectors of the present invention have highly desirable features that permit the high level expression of transgenes in human progenitor cells while meeting human biosafety requirements.

The viral vectors of the present invention, therefore, may be generally described as self-inactivating recombinant vectors that include at least a lentiviral *gag, pol* and *rev* genes, that is, those genes required for virus production, which permit their manufacture in reasonable quantities using available producer cell lines. To meet important human safety needs, the more preferred vectors in accordance with the present invention will not include any other active lentiviral genes, such as *vpr, vif, vpu, nef, tat*, such as where these genes have been removed or otherwise inactivated. In fact, it is preferred that the only active lentiviral genes present in the vector will be the aforementioned *gag, pol* and *rev* genes.

The most preferred lentiviral genes and backbone (*i.e.*, long terminal repeats or LTRs) used in preparing lentivectors in accordance with the present invention will be one that is human immunodeficiency virus (HIV) derived, and more particularly, HIV-1 derived. Thus, the *gag, pol* and *rev* genes will preferably be HIV genes and more preferably HIV-1 genes. However, the *gag, pol* and *rev* genes and LTR regions from other lentiviruses may be employed for certain applications in accordance with the present invention, including the genes and LTRs of HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus, bovine immunodeficiency virus, equine infectious anemia virus, caprine arthritis encephalitis virus and the like. Such constructs could be useful, for example, where one desires to modify certain cells of non-human origin. However, the HIV based vector backbones (*i.e.*, HIV LTR and HIV *gag, pol* and *rev* genes) will generally be preferred in connection with most aspects of the present invention in that HIV-based constructs are the most efficient at transduction of human hematopoietic progenitor cells.

The viral vectors of the present invention will also include an expression cassette comprising a transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a human hematopoietic progenitor cell. To determine whether a particular promoter is useful, a selected promoter is tested in the construct *in vitro* in a selected progenitor cell and, if the promoter is capable of promoting expression of the transgene at a detectable signal-to-noise ratio, it will generally be useful in accordance with the present invention. A desirable signal-to-noise ratio is one between about 10 and about 200, a more desirable signal-to-noise ratio is one 40 and about 200, and an even more desirable signal-to-noise ratio is one between about 150 and about 200. One means of testing such a promoter, described in more detail hereinbelow, is through the use of a signal generating transgene such as the green fluorescent protein (GFP).

Examples of promoters that may be preferably employed in connection with the present invention including an EF1-α, PGK, gp91hox, MHC classII, clotting Factor IX, insulin promoter, PDX1 promoter, CD11, CD4, CD2 or a gp47 promoter. Of these the PGK promoter is preferred, and the EF1-α promoter is particularly preferred. An example of a promoter that is generally not preferred is the CMV promoter, in that this promoter is only minimally active in most progenitor cells. Indeed, it is likely that a CMV promoter will only be useful where one targets human primary B cells or dendritic cells. In any event, however, practice of the present invention is not restricted to the foregoing promoters, so long as the promoter is active in the progenitor, hematopoietic or other cell that one desires to target.

Additionally, the promoters mentioned above can comprise additional elements required for transcription and thus be a part of a transcription cassette. A transcription cassette is defined as comprising one or more promoter elements coupled to enhancers and/or locus control regions, to ensure strong and/or tissue-restricted expression of a transgene.

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It is particularly desirable to employ in the lentivectors of the present invention an LTR region that has reduced promoter activity relative to wild-type LTR, in that such constructs provide a "self-inactivating" (SIN) biosafety feature. Self-inactivating vectors are ones in which the production of full-length vector RNA in transduced cells in greatly reduced or abolished altogether. This feature greatly minimizes the risk that replication-competent recombinants (RCRs) will emerge. Furthermore, it reduces the risk that that cellular coding sequences located adjacent to the vector integration site will be aberrantly expressed. Furthermore, an SIN design reduces the possibility of interference between the LTR and the promoter that is driving the expression of the transgene. It is therefore particularly suitable to reveal the full potential of the internal promoter.

Self-inactivation is preferably achieved through in the introduction of a deletion in the U3 region of the 3' LTR of the vector DNA, *i.e.*, the DNA used to produce the vector RNA. Thus, during reverse transcription, this deletion is transferred to the 5' LTR of the proviral DNA. It is desirable to eliminate enough of the U3 sequence to greatly diminish or abolish altogether the transcriptional activity of the LTR, thereby greatly diminishing or abolishing the production of full-length vector RNA in transduced cells. However, it is generally desirable to retain those elements of the LTR that are involved in polyadenylation of the viral RNA, a function spread out over U3, R and U5. Accordingly, it is desirable to eliminate as many of the transcriptionally important motifs from the LTR as possible while sparing the polyadenylation determinants. In the case of HIV based lentivectors, it has been discovered that such vectors tolerate significant U3 deletions, including the removal of the LTR TATA box (*e.g.*, deletions from –418 to – 18), without significant reductions in vector titers. These deletions render the LTR region substantially transcriptionally inactive in that the transcriptional ability of the LTR in reduced to about 90% or lower. In preferred embodiments the LTR transcription is reduced to about 95% to 99%. Thus, the LTR may be rendered about 90%, 91%, 92%, 93%, 94%, 95% 96% 97%, 98%, to about 99% transcriptionally inactive.

For certain applications, for example, in the case of promoters that are only modestly active in cells targeted for transduction, one will desire to employ a

posttranscriptional regulatory sequence positioned to promote the expression of the transgene. One type of posttranscriptional regulatory sequence is an intron positioned within the expression cassette, which may serve to stimulate gene expression. However, introns placed in such a manner may expose the lentiviral RNA transcript to the normal

5   cellular splicing and processing mechanisms. Thus, in particular embodiments it may be desirable to locate intron-containing transgenes in an orientation opposite to that of the vector genomic transcript.

A more preferred method of enhancing transgene expression is through the use of

10   a posttranscriptional regulatory element which does not rely on splicing events, such as the posttranscriptional processing element of herpes simplex virus, the posttranscriptional regulatory element of the hepatitis B virus (HPRE) or that of the woodchuck hepatitis virus (WPRE), which contains an additional *cis*-acting element not found in the HPRE. The regulatory element is positioned within the vector so as to be included in the RNA

15   transcript of the transgene, but outside of stop codon of the transgene translational unit. It has been found that the use of such regulatory elements are particularly preferred in the context of modest promoters, but may be contraindicated in the case of very highly efficient promoters.

20   It is believed that the lentivectors of the present invention may be employed to deliver any transgene that one desires, depending on the application. In the case of delivery to hematopoietic progenitor cells, one will typically select a transgene that will confer a desirable function on such cells, including, for example, globin genes, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (such

25   as Interleukin-1 (IL-1), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-6 (IL-6), Interleukin-12 (IL-12), *etc.*) and the colony-stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin $\alpha IIb\beta$, multidrug resistance genes, the gp91 or gp 47 genes that are defective in patients with chronic granulomatous

30   disease (CGD), antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation factors VIII or IX

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which are mutated in hemophiliacs, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins) as well as combination of T and B cell antigen receptors alone or in combination with single chain antibodies such as ScFv, tumor necrosis factor (TNF), IL-2, IL-12, gamma interferon, CTLA4, B7 and the like, genes expressed in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100 *etc.*

A principal application of the present transgenes will be to deliver desired transgenes to hematopoietic cells for a number of possible reasons. This might include, but of course not be limited to, the treatment of myelosupression and neutropenias which may be caused as a result of chemotherapy or immunosupressive therapy or infections such as AIDS, genetic disorders, cancers and the like.

Exemplary genetic disorders of hematopoietic cells that are contemplated include sickle cell anemia, thalassemias, hemaglobinopathies, Glanzmann thrombasthenia, lysosomal storage disorders (such as Fabry disease, Gaucher disease, Niemann-Pick disease, and Wiskott-Aldrich syndrome), severe combined immunodeficiency syndromes (SCID), as well as diseases resulting from the lack of systemic production of a secreted protein, for example, coagulation factor VIII and/or IX. In such cases, one would desire to introduce transgenes such as globin genes, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (especially Interleukin-1, Interleukin-2, Interleukin-3, Interleukin-6, Interleukin-12, *etc.*) and the colony-stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin $\alpha IIb\beta$, multidrug resistance genes, the gp91 or gp 47 genes which are defective in patients with chronic granulomatous disease (CGD), antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation factors VIII or IX which are mutated in hemophiliacs, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins), a combination of both T and B cell antigen receptors alone and/or in combination with single chain antibodies (ScFv), IL2, IL12, TNF, gamma

interferon, CTLA4, B7 and the like, genes expressed in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100 *etc.*

Exemplary cancers are those of hematopoietic origin, for example, arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). Lymphoid malignancies which may be treated utilizing the lentivectors of the present invention include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas contemplated as candidates for treatment utilizing the lentiviral vectors of the present invention include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease.

In other embodiments, the present invention is directed to host cells that have been transduced with one of the foregoing lentivectors. It is believed that the lentivectors of the present invention can be employed to transduce most any cell. Exemplary cells include but are not limited to a CD4$^+$ T cell, a peripheral blood lymphocyte cell, a peripheral blood mononuclear cell, a hematopoietic stem cell, a fetal cord blood cell, a fibroblast cell, a brain cell, a lung cell, a liver cell, a muscle cell, a pancreatic cell, an endothelial cell, a cardiac cell, a skin cell, a bone marrow stromal cell, and an eye cells, a pancreatic ductal cell, a neural precursor, a mesodermal stem cell and the like. The cells transduced may further be primate, murine, porcine, or human in origin, or come from another animal species.

For the production of virus particles, one may employ any cell that is compatible with the expression of lentiviral Gag and Pol genes, or any cell that can be engineered to

support such expression. For example, producer cells such as 293T cells and HT1080 cells may be used.

Of course, as noted above, the lentivectors of the invention will be particularly useful in the transduction of human hematopoietic progenitor cell or a hematopoietic stem cell, obtained either from the bone marrow, the peripheral blood or the umbilical cord blood, as well as in the tranduction of a CD4$^+$ T cell, a peripheral blood B or T lymphocyte cell, a peripheral blood mononuclear cell, a dendritic cell, and a monocytic cell. Particularly preferred targets are CD34$^+$ cells.

In still other embodiments, the present invention is directed to a method for transducing a human hematopoietic stem cell comprising contacting a population of human cells that include hematopoietic stem cells with one of the foregoing lentivectors under conditions to effect the transduction of a human hematopoietic progenitor cell in said population by the vector. The stem cells may be transduced *in vivo* or *in vitro*, depending on the ultimate application. Even in the context of human gene therapy, such as gene therapy of human stem cells, one may transduce the stem cell *in vivo* or, alternatively, transduce *in vitro* followed by infusion of the transduced stem cell into a human subject. In one aspect of this embodiment, the human stem cell can be removed from a human, *e.g.*, a human patient, using methods well known to those of skill in the art and transduced as noted above. The transduced stem cells are then reintroduced into the same or a different human.

Where a human subject is treated directly by introduction of the vector into the subject, the treatment is typically carried out by intravenous administration of the vector. When cells, for instance CD34$^+$ cells, dendritic cells, peripheral blood cells or tumor cells are transduced *ex vivo*, the vector particles are incubated with the cells using a dose generally in the order of between 1 to 50 multiplicities of infection (MOI) which also corresponds to 1×10^5 to 50×10^5 transducing units of the viral vector per 10^5 cells. This of course includes amount of vector corresponding to 1, 2, 3, 4, 5, 6, 7, 8 , 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 MOI. Typically, the amount of vector may be expressed in terms

of HeLa transducing units (TU). Other routes for vector administration include intrarterially, endoscopically, intralesionally, percutaneously, subcutaneously, intramuscular, intrathecally, intraorbitally, intradermally, intraperitoneally, transtracheally, subcuticularly, by intrastemal injection, by inhalation or intranasal spraying, by endotracheal route and the like. In embodiments concerning tumor/cancer therapies with the vectors of the invention the expression vector can be delivered by direct injection into the tumor or into the tumor vasculature.

A typical example of *ex vivo* gene therapy is a patient suffering from chronic granulatous disease (CGD), whose CD34$^+$ cells can be isolated from the bone marrow or the peripheral blood and transduced *ex vivo* with a lentivector expressing the gp91hox gene before reimplantation. In the case of patients suffering from severe combined immunodeficiency (SCID), the inventors contemplate a similar approach, using lentivectors of the invention expressing the gene defective in the patient, for example, the gene encoding the common gamma chain of the Interleukin receptor. For the genetic treatment of HIV infection, the present inventors contemplate intracellular immunization, wherein cells are rendered resistant to the HIV virus through the introduction of antiviral genes. In embodiments of the intracellular immunization for HIV, targets of the lentivectors of the invention include hematopoietic progenitors, peripheral blood CD4$^+$ T cells, and monocytes. As will be recognized by the skilled artisan, similar intracellular immunization methods can be used for other viral infections as well. For the immunotherapy of cancers, tumor cells or antigen presenting cells such as dendritic cells will be genetically engineered with the lentivectors of the invention. For cancer therapies some transgenes that may be used in the lentivector constructs of the invention are those that can inhibit, and/or kill, and/or prevent the proliferation, and/or mediate the apoptosis of, the cancer/tumor cell and/or genes such as TNF.

The lentivectors described herein may also be used *in vivo*, by direct injection into the blood or into a specific organ. For example, in one embodiment intracerebral injection of lentivectors expressing the Glial Cell Derived Nerve Growth Factor (GDNF), can be used for the treatment of Parkinson's disease. In another example, intraportal

injection of a lentivector expressing coagulation factor VIII for the correction of hemophilia A is envisioned. In yet another example, intravenous or intramuscular injection of a lentivector of the present invention expressing the dystrophin gene for the treatment of Duchenne Muscular Dystrophy is envisioned. Thus, one of ordinary skill in the art will appreciate the extensive use of the lentivector constructs of the present invention in terms of gene therapies.

As used herein the specification or claim(s) when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A and FIG. 1B. Transgene transfer and expression after transduction of human CD34$^+$ cells with MLV and HIV vectors. Human CD34$^+$ cells from umbilical cord blood (10^5 cells) were transduced with 10^6 HeLa-transducing units (TU) of MLV- or the indicated HIV-derived GFP encoding vectors, previously treated with DNAse I, as described in materials and methods. After 4 days, cells were analyzed by flow cytometry for GFP expression (**FIG. 1A**) and lysed for PCR analysis of the presence of GFP

transgene (**FIG. 1B**). Results from one experiment representative of two independent evaluations are shown. Expression of the GFP transgene after transduction of human CD34$^+$ cells or HeLa cells. Results are represented as histograms of GFP fluorescence intensity (x-axis, four-log scale) versus cell number (y-axis, linear). GFP$^+$ cells (within marker) were analyzed for percentage (lower number) and median of fluorescence intensity (upper number). Percentages were omitted in HeLa cells since the histograms were obtained from titration experiments of the corresponding vectors **(FIG. 1A)**. Presence of the GFP transgene after transduction and expansion of human CD34$^+$ cells. Cellular extracts equivalent to 5000 cells (upper panel) were amplified as described in the section entitled Examples with GFP-specific primers, together with IL-2 specific primers as internal controls. Sizes of the corresponding PCR products are indicated. Lanes are: M, molecular weight marker; HeLa (negative control for GFP, positive control for IL-2); 4.5, a clone of HeLa containing one copy of HIV-CMV-GFP vector; 0, untransduced CD34$^+$ cells; MLV-CMV-PGK-EF1, CD34$^+$ cells transduced with the corresponding vectors (see FIG. 2A); CMV-EPO, CD34$^+$ cells transduced with HIV-CMV vector (lane CMV) and analyzed after expansion and differentiation into erythroid cells (see text); CMV-GM, same as CMV-EPO but analyzed after expansion and differentiation into monocytic cells (see text); PGK-EPO and PGK-GM, same as CMV-EPO and CMV-GM but from CD34+ cells transduced with HIV-CMV vector (lane PGK). To ensure for proportionality, cellular extracts equivalent to 1700 cells (lower panel) were amplified separately **(FIG. 1B).**

FIG. 2. Effect of MOI on transduction efficiency of lentivectors in human CD34$^+$ cells. 10^5 CD34$^+$ cells were transduced with various doses (1, 2, 5, 10, 20 and 50x10^5 TU) of EF1α-GFP lentiviral vector allowing multiplicities of infection (MOI) ranging from 1 to 50. GFP expression was analyzed by flow cytometry after 4 days. Upper panel: Data from one representative experiment are shown as frequency histograms of GFP fluorescence intensity versus cell number (events). Gates for GFP$^+$ cells were setup according to untransfected cells (MOI 0). Lower panel: Mean percentages of GFP$^+$ cells obtained with MOIs of 0 to 10 and 10 to 50 are represented in the main panel and in the

insert respectively. These data are from 3 independent experiments for the 0-10 MOI range and from 2 for 10-50 MOI range. Error bars represent SD.

FIG. 3. Transgene expression in differentiated hematopoietic lineages after transduction of human CD34$^+$ cells with HIV-derived vectors. Human CD34$^+$ cells from umbilical cord blood (10^5 cells) were transduced with 10^6 TU of HIV vectors (corresponding to a MOI of 10) containing either the EF1α or the PGK promoters and differentiated into various hematopoietic lineages as described in the section entitled Examples. After differentiation, HIV-EF1α- (first and second column) or HIV-PGK-transduced cells (third column) were analyzed by flow cytometry for both GFP (x axis) and lineage-specific markers expression (glycophorin, CD14, CD42b, CD15 and CD1a for respectively erythroids, monocytes, megakaryocytes, granulocytes, and dendritic cells). Isotype control antibodies were used in the first column. For each promoter, cell populations expressing high levels of lineage-specific marker were gated (upper rectangle in 2D plots), and monoparametric frequency histograms of GFP expression for these cells were generated (located above the 2D plots). The percentage of GFP$^+$ cells (upper number), and median of fluorescence intensity of GFP (lower number) was determined. For the experiment generating erythroids, monocytes, megakaryocytes and granulocytes, the fraction of GFP$^+$ CD34$^+$ cells before differentiation was 22% for EF1α and 16% for PGK. For the experiment generating DCs, the fraction of GFP$^+$ precursors before differentiation was 32% for EF1α and 29% for PGK. These data are representative of 4 independent experiments.

FIG. 4. GFP expression in lentivector-transduced primary human T lymphocytes. CMV-, PGK- or EF1α-containing HIV-derived vectors were used to infect PHA-activated primary T lymphocytes. Cells were maintained for 5 days in culture medium supplemented with IL-2 before analyzing GFP expression by flow cytometry. Two-dimensional plot represents cell number as a function of GFP levels. Results are representative of at least five independent experiments.

FIG. 5A. and FIG. 5B. Effect of SIN design and WPRE addition on transgene expression in human CD34$^+$ cells. 10^5 CD34$^+$ cells were transduced with 10^6 HeLa-TU of the indicated GFP-expressing HIV-derived vectors and analyzed by flow cytometry four days later. Vectors were as follows: **(FIG. 5A)** PGK (2 intact LTRs); PGK-SIN (3'SIN LTR deletion, see materials and methods); PGK-SIN-W (addition of WPRE upstream of the 3'SIN-LTR). **(FIG. 5B)** Same analysis as in FIG. 5A, but with EF1α promoter instead of PGK promoter. Results are represented as contour graphs of CD34 expression versus GFP expression (four-log scale). For each condition, GFP expression is also displayed as histograms of GFP fluorescence intensity (x-axis, four-log scale) versus number of cells (y-axis, linear). Number in square represents median of fluorescence intensity of GFP$^+$ cells.

FIG. 6. Map depicting the construct pHR-CMV-GFP. Same as pHR-CMV-LACz (Genbank AF 105229) but with EGFP instead of LacZ.

FIG. 7. Map depicting the construct pHR-EF1-GFP. Also referred to as pHR'-EF1alpha-EGFP. *ClaI, HindIII* and *EcoRI* cut outside of EF1alpha promoter (Nagata, G181962).

FIG. 8. Map depicting the construct pHR-EF1-GFP-SIN. Deletion of WPRE by insertion of Asp718/Asp718 fragment (~0.8kb) from pHR'-EFI alpha-EGFP (Uni-Lund Lab, Sweden) into Asp718/Asp718 sites of pHR-E-GFP-W.

FIG. 9. Map depicting the construct pHR-EF1-GFP-W-SIN. Insertion of ClaI/BamHI fragment from pHR'-EF1-GFP (Uni-Lund, Sweden) containing the promoter of the human EF1 alpha gene (Nagata, G181962) into ClaI/BamHI sites of pHRGFP/delta ClaI. SalI site at 1757 destroyed for subsequent clonings.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

While lentiviral vectors offer a great potential for gene-therapy and especially the transduction of human hematopoietic stem cells (hHSC), vectors developed so far have failed to meet biosaftey standards and are still inefficient in expression of transgenes. For example, while CMV promoter-containing HIV-derived vectors can induce high levels of transgene expression in the central nervous system (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b; Blomer *et al.*, 1997), and allowed the initial demonstration that pluripotent hematopoietic precursors can be efficiently transduced by this gene delivery tool, they are largely useless for transferring therapeutic genes into most lympho-hematopoietic cells, because in these targets their transcriptional activity is prohibitively low (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000). Current lentiviral vectors have multiply attenuated HIV virulence genes which removes the potential for reconstitution of wild-type virus by recombination (Zufferey *et al.*, 1997; Dull *et al.*, 1998). A self-inactivating design rendered the vectors further biologically safe by eliminating the transcriptional elements of HIV (Zufferey *et al.*, 1998). However, this can negatively affect transgene expression, apparently by decreasing the efficiency of polyadenylation (DeZazzo *et al.*, 1991; Valsamakis *et al.*, 1991; Brown *et al.*, 1991; Cherrington and Ganem, 1992; Valsamakis *et al.*, 1992; Gilmartin *et al.*, 1992).

The present invention overcomes such and other deficiencies in the art and describes the development of improved HIV-derived vectors that are optimized in terms of both biosaftey and increased gene expression. Thus, the transduction of human cells with HIV-derived lentivectors which comprise elements that prevent the formation of replication competent recombinants (RCR) and further comprise an internal promoter element which induces high levels of transgene expression in both hematopoietic precursors and *in vitro* differentiated blood lineages, as well as in primary T cells demonstrated herein that. For example, human CD34$^+$ cells as well as other human hematopoietic lineages can be transduced using the vectors of this invention.

The promoter elements of the vectors described include the EF1α promoter or the PGK promoter, although, as will be recognized by one of skill in the art, almost any promoter element may be used. However, CMV promoters are not preferred. For example, the inventors demonstrate using the green fluorescent protein (GFP) expression that while the EF1α promoter element or the PGK promoter were highly active in CD34$^+$ cells as well as several other differentiated hematopoietic derivatives, the CMV promoter driven GFP production was insufficient to determine either the percentage of transduced cells or the level of transgene expression in those cells. Also contemplated are engraftment and repopulation assays in NOD/SCID mice with both HIV-PGK and HIV-EF1α vectors, to confirm the stability of expression from these promoters *in vivo*.

The element that prevents RCR in the lentivectors of the present invention is the self inactivating (SIN) design. This is achieved by the deletion of a major part of U3 in the 3'LTR of the vector plasmid, leading to a self-inactivating (SIN) configuration (Zufferey *et al.*, 1998). This deletion also prevents potential interference between LTR and the internal promoter elements. However, the SIN can induce decreases in transgene expression, especially in promoters that are not very strong such as the PGK promoter. The invention further describes methods that rescue transgene levels in lentivector constructs that do not have strong promoters by inserting other regulatory elements such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) or the hepatitis virus B regulatory element (HPRE) in the vector, immediately upstream of the deleted 3' LTR.

The lentivectors of this invention can efficiently transduce several human blood lineage cells including CD34$^+$ cells using conditions under which MLV-based vectors are inefficient. Furthermore, it is also demonstrated that human CD34$^+$ cells can be efficiently transduced at a relatively low MOI, although the efficacy of gene transfer saturates at about 20 to 30% of transduced cells. For example, an MOI of 10 was used to achieve optimal transduction which is significantly lower than that described in previous studies, where it ranged between 60-300 and 1000-3000 (Miyoshi *et al.*, 1999; Case *et al.*, 1999). This may in part be due to enhanced vector-target meeting probability as the

methods of the present invention involve exposure of CD34$^+$ cells to the vector particles in a small volume (10^5 cells in 200 μl) and for a duration of 24 hrs.

Thus, the present invention provides HIV-derived vectors which are safe, highly
5   efficient, and very potent for expressing transgenes in human hematopoietic progenitor cells as well as in all other blood cell derivatives, even in a self-inactivating configuration. These vectors therefore provide useful tools for genetic treatments such as inherited and acquired lympho-hematological disorders, gene-therapies for cancers especially the hematological cancers, as well as for the study of hematopoiesis *via*
10   lentivector-mediated modification of human HSCs.

A.   Lentiviral Vectors and Gene Therapy

Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes *gag, pol,* and *env,* contain other genes with regulatory or structural
15   function. The higher complexity enables the virus to modulate its life cycle, as in the course of latent infection. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes *env, vif, vpr, vpu* and *nef* are deleted making the vector
20   biologically safe.

Lentiviral vectors offer great advantages for gene therapy. They integrate stably into chromosomes of target cells which is required for long-term expression. Further, they do not transfer viral genes therefore avoiding the problem of generating transduced
25   cells that can be destroyed by cytotoxic T-cells. Furthermore, they have a relatively large cloning capacity, sufficient for most envisioned clinical applications. In addition, lentiviruses, in contrast to other retroviruses, are capable of transducing non-dividing cells. This is very important in the context of gene-therapy for tissues such as the hematopoietic system, the brain, liver, lungs and muscle. For example, vectors derived
30   from HIV-1 allow efficient *in vivo* and *ex vivo* delivery, integration and stable expression

of transgenes into cells such a neurons, hepatocytes, and myocytes (Blomer *et al.*, 1997; Kafri *et al.*, 1997; Naldini *et al.*, 1996; Naldini *et al.*, 1998).

The lentiviral genome and the proviral DNA have the three genes found in retroviruses: *gag, pol* and *env*, which are flanked by two long terminal repeat (LTR) sequences.   The *gag* gene encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the *pol* gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease and an integrase; and the *env* gene encodes viral envelope glycoproteins.   The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other *cis*-acting sequences necessary for viral replication. Lentiviruses have additional genes including *vif, vpr, tat, rev, vpu, nef* and *vpx.*

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the *Psi* site).   If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the *cis* defect prevents encapsidation of genomic RNA. However, the resulting mutant remains capable of directing the synthesis of all virion proteins.

Lentiviral vectors are known in the art, see Naldini *et al.*, (1996 and 1998); Zufferey *et al.*, (1997); Dull *et al.*, 1998, U.S. Pat. Nos. 6,013,516;and 5,994,136 all incorporated herein by reference. In general, these vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection and for transfer of the nucleic acid into a host cell.

Two components are involved in making a virus-based gene delivery system: first, the packaging elements, encompassing the structural proteins as well as the enzymes necessary to generate an infectious particle, and second, the vector itself, i.e., the genetic material to be transferred. Biosaftey safeguards can be introduced in the design of both of these components. Thus, the packaging unit of the first generation HIV-based vectors

comprised all HIV-1 proteins except the envelope proteins (Naldini *et al.*, 1998). Subsequently it was shown that the deletion of four additional viral genes that are responsible for virulence including, *vpr, vif, vpu* and *nef* did not alter the utility of the vector system (Zufferey *et al.*, 1997). It was also shown that Tat, the main transactivator of HIV is also dispensable for the generation of a fully efficient vector (Dull *et al.*, 1998). Thus, the third-generation packaging unit of the HIV-based lentiviral vectors comprise only three genes of the parental virus: *gag, pol* and *rev*, which eliminates the possibility of reconstitution of a wild-type virus through recombination.

This system was further improved by removing HIV transcriptional units from the vector (Zufferey *et al.*, 1998). It was demonstrated therein that introducing a deletion in the U3 region of the 3' LTR of the DNA used to produce the vector RNA generated self-inactivating (SIN) vectors. During reverse transcription this deletion is transferred to the 5' LTR of the proviral DNA. Enough sequence was eliminated, including the removal of a TATA box, which abolished the transcriptional activity of the LTR, which prevents production of full-length vector RNA in transduced cells. This however did not affect vector titers or the *in vitro* or *in vivo* properties of the vector.

The present invention provides several improvements to the existing lentivectors as described above and in other parts of this specification. Introducing a lentivector providing a heterologous gene, such as genes to treat hematopoietic and lympho-hematopoietic disorders in this invention, into a packaging cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest.

The *env* gene can be derived from any virus, including retroviruses. The *env* preferably is an amphotropic envelope protein which allows transduction of cells of human and other species. Examples of retroviral-derived *env* genes include, but are not limited to: Moloney murine leukemia virus (MoMuLV or MMLV), Harvey murine sarcoma virus (HaMuSV or HSV), murine mammary tumor virus (MuMTV or MMTV), gibbon ape leukemia virus (GaLV or GALV), human immunodeficiency virus (HIV) and

Rous sarcoma virus (RSV). Other *env* genes such as Vesicular stomatitis virus (VSV) protein G (VSV G), that of hepatitis viruses and of influenza also can be used.

While VSV G protein is a desirable *env* gene because VSV G confers broad host range on the recombinant virus, VSV G can be deleterious to the host cell. Thus, when a gene such as that for VSV G is used, it is preferred to employ an inducible promoter system so that VSV G expression can be regulated to minimize host toxicity when VSV G is expression is not required. For example, the tetracycline-regulatable gene expression system of Gossen & Bujard, (1992) can be employed to provide for inducible expression of VSV G when tetracycline is withdrawn from the transferred cell. Thus, the tet/VP16 transactivator is present on a first vector and the VSV G coding sequence is cloned downstream from a promoter controlled by tet operator sequences on another vector.

The vector providing the viral *env* nucleic acid sequence is associated operably with regulatory sequences, e.g., a promoter or enhancer. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, EF1α, PGK, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus enhancer, the vaccinia P7.5 promoter or the like (also see examples listed in Tables 1 and 2 below). In some cases, such as the Moloney murine leukemia virus promoter-enhancer element, the promoter-enhancer elements are located within or adjacent to the LTR sequences. Preferably, the regulatory sequence is one which is not endogenous to the lentivirus from which the vector is being constructed. Thus, if the vector is being made from SIV, the SIV regulatory sequence found in the SIV LTR would be replaced by a regulatory element which does not originate from SIV.

One may further target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific. Retroviral vectors can be made target-

specific by inserting, for example, a glycolipid or a protein. Targeting often is accomplished by using an antigen-binding portion of an antibody or a recombinant antibody-type molecule, such as a single chain antibody, to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a retroviral vector to a specific target.

The heterologous or foreign nucleic acid sequence, such as a polynucleotide sequence encoding a gene such as a therapeutic gene for inherited or acquired hematopoietic disorders herein, is linked operably to a regulatory nucleic acid sequence. Preferably, the heterologous sequence is linked to a promoter, resulting in a chimeric gene.

Marker genes may be utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of a marker gene ensures the selection and growth of only those host cells which express the inserts. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, *e.g.,* histidinol, puromycin, hygromycin, neomycin, methotrexate, and cell surface markers.

The recombinant virus of the invention is capable of transferring a nucleic acid sequence into a mammalian cell. The term, "nucleic acid sequence", refers to any nucleic acid molecule, preferably DNA, as discussed in detail herein. The nucleic acid molecule may be derived from a variety of sources, including DNA, cDNA, synthetic DNA, RNA or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, poly A sequences or other associated sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription or other means.

The vectors are introduced via transfection or infection into the packaging cell line. The packaging cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After cotransfection of the packaging vectors and the transfer vector to the packaging cell line, the recombinant virus is recovered from the culture media and tittered by standard methods used by those of skill in the art. Thus, the packaging constructs can be introduced into human cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neomycin, DHFR, Glutamine synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. The selectable marker gene can be linked physically to the packaging genes in the construct.

Stable cell lines wherein the packaging functions are configured to be expressed by a suitable packaging cell are known. For example, see U.S. Pat. No. 5,686,279; and Ory et al., (1996), which describe packaging cells. The packaging cells with a lentiviral vector incorporated in them form producer cells. Producer cells are thus cells or cell-lines that can produce or release packaged infectious viral particles carrying the therapeutic gene of interest. These cells can further be anchorage dependent which means that these cells will grow, survive, or maintain function optimally when attached to a surface such as glass or plastic. The producer cells may also be neoplastically transformed cells. Some examples of anchorage dependent cell lines used as lentiviral vector packaging cell lines when the vector is replication competent are HeLa or 293 cells and PERC.6 cells.

In some applications, particularly when the virus is to be used for gene therapy applications, it is preferable that the vector be replication deficient (or replication defective) to avoid uncontrolled proliferation of the virus in the individual to be treated. In such instances mammalian cell lines are selected which have been engineered, either by modification of the producer cell's genome to encode essential viral functions or by the co-infection of the producer cell with a helper virus, to express proteins complementing the effect of the sequences deleted from the viral genome. For example,

for HIV-1 derived vectors, the HIV-1 packaging cell line, PSI422, may be used as described in Corbeau, *et al.* (1996). Similarly, where the viral vector to be produced is a retrovirus, the human 293-derived retroviral packaging cell line (293GPG) capable of producing high titers of retroviral particles may be employed as described in Ory, *et al.* (1996). In the production of minimal vector systems, the producer cell is engineered (either by modification of the viral genome or by the use of helper virus or cosmid) to complement the functions of the parent virus enabling replication and packaging into virions in the producer cell line.

Lentiviral transfer vectors Naldini *et al.*, (1996), have been used to infect human cells growth-arrested *in vitro* and to transduce neurons after direct injection into the brain of adult rats. The vector was efficient at transferring marker genes *in vivo* into the neurons and long term expression in the absence of detectable pathology was achieved. Animals analyzed ten months after a single injection of the vector showed no decrease in the average level of transgene expression and no sign of tissue pathology or immune reaction (Blomer *et al.*, (1997).

B. The SIN Design

The SIN design increases the biosaftey of the lentiviral vectors. The majority of the HIV LTR is comprised of the U3 sequences. The U3 region contains the enhancer and promoter elements that modulate basal and induced expression of the HIV genome in infected cells and in response to cell activation. Several of these promoter elements are essential for viral replication. Some of the enhancer elements are highly conserved among viral isolates and have been implicated as critical virulence factors in viral pathogenesis. The enhancer elements may act to influence replication rates in the different cellular target of the virus (Marthas *et al.*, 1993).

As viral transcription starts at the 3' end of the U3 region of the 5' LTR, those sequences are not part of the viral mRNA and a copy thereof from the 3' LTR acts as template for the generation of both LTR's in the integrated provirus. If the 3' copy of the U3 region is altered in a retroviral vector construct, the vector RNA is still produced from

the intact 5' LTR in producer cells, but cannot be regenerated in target cells. Transduction of such a vector results in the inactivation of both LTR's in the progeny virus. Thus, the retrovirus is self-inactivating (SIN) and those vectors are known as SIN transfer vectors.

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The SIN design is described in further detail in Zufferey *et al.*, 1998 and U.S. Pat. No. 5,994,136 both incorporated herein by reference. As described therein, there are, however, limits to the extent of the deletion at the 3' LTR. First, the 5' end of the U3 region serves another essential function in vector transfer, being required for integration (terminal dinucleotide+att sequence). Thus, the terminal dinucleotide and the att sequence may represent the 5' boundary of the U3 sequences which can be deleted. In addition, some loosely defined regions may influence the activity of the downstream polyadenylation site in the R region. Excessive deletion of U3 sequence from the 3' LTR may decrease polyadenylation of vector transcripts with adverse consequences both on the titer of the vector in producer cells and the transgene expression in target cells. On the other hand, limited deletions may not abrogate the transcriptional activity of the LTR in transduced cells.

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The lentiviral vectors described herein carry deletions of the U3 region of the 3' LTR spanning from nucleotide -418 to -18. This is the most extensive deletion and extends as far as to the TATA box, therefore abrogating any transcriptional activity of the LTR in transduced cells. The titer of vector in producer cells as well as transgene expression in target cells was unaffected in these vectors. This design therefore provides an enormous increase in vector safety.

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SIN-type vectors with such extensive deletions of the U3 region cannot be generated for murine leukemia virus (MLV) or spleen necrosis virus (SNV) based retroviral vectors without compromising efficiency of transduction.

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Elimination of the −418 to −18 nucleotide sequence abolishes transcriptional activity of the LTR, thereby abolishing the production of full length vector RNA in

transduced cells. In the HIV-derived lentivectors none of the *in vitro* or *in vivo* properties were compromised by the SIN design.

C.   Posttranscriptionally Regulating Elements (PRE)

Enhancing transgene expression may be required in certain embodiments, especially those that involve lentiviral constructs of the present invention with modest promoters.

One type of PRE is an intron positioned within the expression cassette, which can stimulate gene expression. However, introns can be spliced out during the life cycle events of a lentivirus. Hence, if introns are used as PRE's they have to be placed in an opposite orientation to the vector genomic transcript.

Posttranscriptional regulatory elements that do not rely on splicing events offer the advantage of not being removed during the viral life cycle. Some examples are the posttranscriptional processing element of herpes simplex virus, the posttranscriptional regulatory element of the hepatitis B virus (HPRE) and the woodchuck hepatitis virus (WPRE). Of these the WPRE is most preferred as it contains an additional *cis*-acting element not found in the HPRE (Donello *et al.*, 1998). This regulatory element is positioned within the vector so as to be included in the RNA transcript of the transgene, but outside of stop codon of the transgene translational unit. As demonstrated in the present invention and in Zufferey *et al.*, 1999, the WPRE element is a useful tool for stimulating and enhancing gene expression of desired transgenes in the context of the lentiviral vectors.

The WPRE is characterized and described in U.S. Pat. No. 6,136,597, incorporated herein by reference. As described therein, the WPRE is an RNA export element that mediates efficient transport of RNA from the nucleus to the cytoplasm. It enhances the expression of transgenes by insertion of a *cis*-acting nucleic acid sequence, such that the element and the transgene are contained within a single transcript. Presence of the WPRE in the sense orientation was shown to increase transgene expression by up

to 7 to 10 fold. Retroviral vectors transfer sequences in the form of cDNAs instead of complete intron-containing genes as introns are generally spliced out during the sequence of events leading to the formation of the retroviral particle. Introns mediate the interaction of primary transcripts with the splicing machinery. Because the processing of RNAs by the splicing machinery facilitates their cytoplasmic export, due to a coupling between the splicing and transport machineries, cDNAs are often inefficiently expressed. Thus, the inclusion of the WPRE in a vector results in enhanced expression of transgenes.

D. Nucleic Acids

One embodiment of the present invention is to transfer nucleic acids encoding a therapeutic gene, especially a gene that provides therapy for hematopoietic and lympho-hematopoietic disorders, such as the inherited or acquired disorders described above. In one embodiment the nucleic acids encode a full-length, substantially full-length, or functional equivalent form of such a gene.

Thus, in some embodiments of the present invention, the treatment of a hematopoietic and lympho-hematopoietic disorder involves the administration of a lentiviral vector of the invention comprising a therapeutic nucleic acid expression construct to a cell of hematopoietic origin. It is contemplated that the hematopoietic cells take up the construct and express the therapeutic polypeptide encoded by nucleic acid, thereby restoring the cells normal phenotype.

A nucleic acid may be made by any technique known to one of ordinary skill in the art. Non-limiting examples of synthetic nucleic acid, particularly a synthetic oligonucleotide, include a nucleic acid made by *in vitro* chemical synthesis using phosphotriester, phosphite or phosphoramidite chemistry and solid phase techniques such as described in EP 266,032, incorporated herein by reference, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, 1986, and U.S. Patent Serial No. 5,705,629, each incorporated herein by reference. A non-limiting example of enzymatically produced nucleic acid include one produced by enzymes in amplification reactions such as PCR™ (see for example, U.S. Patent 4,683,202 and U.S. Patent

4,682,195, each incorporated herein by reference), or the synthesis of oligonucleotides described in U.S. Patent No. 5,645,897, incorporated herein by reference. A non-limiting example of a biologically produced nucleic acid includes recombinant nucleic acid production in living cells (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

The term "nucleic acid" will generally refer to at least one molecule or strand of DNA, RNA or a derivative or mimic thereof, comprising at least one nucleobase, such as, for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.,* adenine "A," guanine "G," thymine "T," and cytosine "C") or RNA (*e.g.* A, G, uracil "U," and C). The term "nucleic acid" encompasses the terms "oligonucleotide" and "polynucleotide." The term "oligonucleotide" refers to at least one molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length. These definitions generally refer to at least one single-stranded molecule, but in specific embodiments will also encompass at least one additional strand that is partially, substantially or fully complementary to the at least one single-stranded molecule. Thus, a nucleic acid may encompass at least one double-stranded molecule or at least one triple-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a strand of the molecule.

In certain embodiments, a "gene" refers to a nucleic acid that is transcribed. As used herein, a "gene segment" is a nucleic acid segment of a gene. In certain aspects, the gene includes regulatory sequences involved in transcription, or message production or composition. In particular embodiments, the gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. In other particular aspects, the gene comprises a nucleic acid, and/or encodes a polypeptide or peptide-coding sequences of a

gene that is defective or mutated in a hematopoietic and lympho-hematopoietic disorder. In keeping with the terminology described herein, an "isolated gene" may comprise transcribed nucleic acid(s), regulatory sequences, coding sequences, or the like, isolated substantially away from other such sequences, such as other naturally occurring genes, regulatory sequences, polypeptide or peptide encoding sequences, etc. In this respect, the term "gene" is used for simplicity to refer to a nucleic acid comprising a nucleotide sequence that is transcribed, and the complement thereof. In particular aspects, the transcribed nucleotide sequence comprises at least one functional protein, polypeptide and/or peptide encoding unit. As will be understood by those in the art, this functional term "gene" includes both genomic sequences, RNA or cDNA sequences, or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or enhancer regions of a gene. Smaller engineered gene nucleic acid segments may express, or may be adapted to express using nucleic acid manipulation technology, proteins, polypeptides, domains, peptides, fusion proteins, mutants and/or such like. Thus, a "truncated gene" refers to a nucleic acid sequence that is missing a stretch of contiguous nucleic acid residues.

Various nucleic acid segments may be designed based on a particular nucleic acid sequence, and may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all nucleic acid segments can be created:

$$n \text{ to } n + y$$

where n is an integer from 1 to the last number of the sequence and y is the length of the nucleic acid segment minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the nucleic acid segments correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and/or so on. For a 15-mer, the nucleic acid segments correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and/or so on. For a 20-mer, the nucleic segments correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and/or so on.

The nucleic acid(s) of the present invention, regardless of the length of the sequence itself, may be combined with other nucleic acid sequences, including but not limited to, promoters, enhancers, polyadenylation signals, restriction enzyme sites, multiple cloning sites, coding segments, and the like, to create one or more nucleic acid

5   construct(s). The overall length may vary considerably between nucleic acid constructs. Thus, a nucleic acid segment of almost any length may be employed, with the total length preferably being limited by the ease of preparation or use in the intended recombinant nucleic acid protocol.

10   The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. Vectors of the present invention are lentivirus based as described above and in other parts of the specification. The nucleic acid molecules carried by the vectors of the invention encode therapeutic genes and will be used for carrying out gene-therapies.

15   One of skill in the art would be well equipped to construct such a therapeutic vector through standard recombinant techniques (see, for example, Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both incorporated herein by reference).

The term "expression vector" refers to any type of genetic construct comprising a

20   nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an

25   operably linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described below.

(a)   **Promoters and Enhancers**

30   A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements

at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a *cis*-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding

segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the β-lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patents 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well. Control sequences comprising promoters, enhancers and other locus or transcription controlling/modulating elements are also referred to as "transcriptional cassettes".

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (see, for example Sambrook *et al.*, 1989, incorporated herein by reference). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous for gene therapy or for applications such as the large-

scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Tables 1 lists non-limiting examples of elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a RNA. Table 2 provides non-limiting examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 1	
Promoter and/or Enhancer	
Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl *et al.*, 1985; Atchinson *et al.*, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen *et al.*, 1983; Picard *et al.*, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto *et al.*, 1989; Redondo *et al.*; 1990
HLA DQ a and/or DQ β	Sullivan *et al.*, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn *et al.*, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase (MCK)	Jaynes *et al.*, 1988; Horlick *et al.*, 1989; Johnson *et al.*, 1989

TABLE 1	
Promoter and/or Enhancer	
Promoter/Enhancer	References
Prealbumin (Transthyretin)	Costa et al., 1988
Elastase I	Omitz et al., 1987
Metallothionein (MTII)	Karin et al., 1987; Culotta et al., 1989
Collagenase	Pinkert et al., 1987; Angel et al., 1987
Albumin	Pinkert et al., 1987; Tronche et al., 1989, 1990
α-Fetoprotein	Godbout et al., 1988; Campere et al., 1989
γ-Globin	Bodine et al., 1987; Perez-Stable et al., 1990
β-Globin	Trudel et al., 1987
c-fos	Cohen et al., 1987
c-HA-ras	Triesman, 1986; Deschamps et al., 1985
Insulin	Edlund et al., 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh et al., 1990
α1-Antitrypain	Latimer et al., 1990
H2B (TH2B) Histone	Hwang et al., 1990
Mouse and/or Type I Collagen	Ripe et al., 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang et al., 1989
Rat Growth Hormone	Larsen et al., 1986
Human Serum Amyloid A (SAA)	Edbrooke et al., 1989
Troponin I (TN I)	Yutzey et al., 1989
Platelet-Derived Growth Factor (PDGF)	Pech et al., 1989
Duchenne Muscular Dystrophy	Klamut et al., 1990
SV40	Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., 1985; Firak et al., 1986; Herr et al., 1986; Imbra et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988

TABLE 1	
Promoter and/or Enhancer	
Promoter/Enhancer	**References**
Polyoma	Swartzendruber *et al.*, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman *et al.*, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and/or Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987
Hepatitis B Virus	Bulla *et al.*, 1986; Jameel *et al.*, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et al.*, 1989
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2		
Inducible Elements		
Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter et al., 1982; Haslinger et al., 1985; Searle et al., 1985; Stuart et al., 1985; Imagawa et al., 1987, Karin et al., 1987; Angel et al., 1987b; McNeall et al., 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang et al., 1981; Lee et al., 1981; Majors et al., 1983; Chandler et al., 1983; Lee et al., 1984; Ponta et al., 1985; Sakai et al., 1988
β-Interferon	Poly(rI)x Poly(rc)	Tavernier et al., 1983
Adenovirus 5 E2	ElA	Imperiale et al., 1984
Collagenase	Phorbol Ester (TPA)	Angel et al., 1987a
Stromelysin	Phorbol Ester (TPA)	Angel et al., 1987b
SV40	Phorbol Ester (TPA)	Angel et al., 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug et al., 1988
GRP78 Gene	A23187	Resendez et al., 1988
α-2-Macroglobulin	IL-6	Kunz et al., 1989
Vimentin	Serum	Rittling et al., 1989
MHC Class I Gene H-2κb	Interferon	Blanar et al., 1989
HSP70	ElA, SV40 Large T Antigen	Taylor et al., 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq et al., 1989
Tumor Necrosis Factor	PMA	Hensel et al., 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee et al., 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Non-limiting examples of such regions include the human LIMK2 gene (Nomoto *et al.*, 1999), the

5   somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al.*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), and human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996).

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The lentiviral vectors of the present invention are designed, primarily, to transform cells with a therapeutic gene under the control of regulated eukaryotic promoters. Although the EF1α -promoter and the PGK promoter are preferred other promoter and regulatory signal elements as described in the Tables 1 and 2 above may

15   also be used. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding the therapeutic gene of interest that is used in context with the lentiviral vectors of the present invention. Alternatively, a tissue-specific promoter for cancer gene therapy or the targeting of tumors may be employed with the lentiviral vectors of the present

20   invention for treatment of cancers, especially hematological cancers.

Typically promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by

25   each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA.

30   This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA

with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

A signal that may prove useful is a polyadenylation signal (hGH, BGH, SV40). The use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'–methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well as an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

In any event, it will be understood that promoters are DNA elements which when positioned functionally upstream of a gene leads to the expression of that gene. Most transgenes that will be transformed using the lentiviral vectors of the present invention are functionally positioned downstream of a promoter element.

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A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

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(b)   Multiple Cloning Sites

Vectors of the present invention can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

(c)   Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts.   Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, Chandler *et al.*, 1997, herein incorporated by reference.)

(d)   Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for

example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

(e)   Polyadenylation Signals

In eukaryotic gene expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Some examples include the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

(f)   Origins of Replication

In order to propagate a vector of the invention in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

(g)   Selectable and Screenable Markers

In certain embodiments of the invention, cells transduced with the lentivectors of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the transduced cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genetic constructs that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful

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selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

E.   Host Cells

As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organisms that is capable of replicating a vector and/or expressing a heterologous nucleic acid encoded by the vectors of this invention. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous nucleic acid sequence, such as, for example, a lentivector of the invention bearing a therapeutic gene construct, has been introduced. Therefore, recombinant cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced nucleic acid.

In certain embodiments, it is contemplated that RNAs or proteinaceous sequences may be co-expressed with other selected RNAs or proteinaceous sequences in the same host cell. Co-expression may be achieved by co-transfecting the host cell with two or

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more distinct recombinant vectors. Alternatively, a single recombinant vector may be constructed to include multiple distinct coding regions for RNAs, which could then be expressed in host cells transfected with the single vector.

5   Host cells may be derived from prokaryotes or eukaryotes, depending upon whether the desired result is replication of the vector or expression of part or all of the vector-encoded nucleic acid sequences. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures

10   and genetic materials (www.atcc.org). Some examples of host cells used in this invention include but are not limited to virus packaging cells, virus producer cells, 293T cells, human hematopoietic progenitor cells, human hematopoietic stem cells, CD34$^+$ cells CD4$^+$cells, and the like.

15   **(a)   Tissues and Cells**

A tissue may comprise a host cell or cells to be transformed or contacted with a nucleic acid delivery composition and/or an additional agent. The tissue may be part or separated from an organism. In certain embodiments, a tissue and its constituent cells may comprise, but is not limited to, blood (*e.g.*, hematopoietic cells (such as human

20   hematopoietic progenitor cells, human hematopoietic stem cells, CD34$^+$ cells CD4$^+$cells), lymphocytes and other blood lineage cells), bone marrow, brain, stem cells, blood vessel, liver, lung, bone, breast, cartilage, cervix, colon, cornea, embryonic, endometrium, endothelial, epithelial, esophagus, facia, fibroblast, follicular, ganglion cells, glial cells, goblet cells, kidney, lymph node, muscle, neuron, ovaries, pancreas, peripheral blood,

25   prostate, skin, skin, small intestine, spleen, stomach, testes.

(b)   Organisms

In certain embodiments, the host cell or tissue may be comprised in at least one organism. In certain embodiments, the organism may be, human, primate or murine. In

30   other embodiments the organism may be any eukaryote or even a prokayote (*e.g.*, a eubacteria, an archaea), as would be understood by one of ordinary skill in the art (see,

for example, webpage http://phylogeny.arizona.edu/tree/phylogeny.html). Some lentivectors of the invention may employ control sequences that allow them to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of the lentivectors of the invention, as well as production of the nucleic acids encoded by the lentivectors and their cognate polypeptides, proteins, or peptides some of which are therapeutic genes or proteins which will be used for gene therapies.

F. Injectable Compositions and Pharmaceutical Formulations

To achieve gene-therapy using the lentiviral vector compositions of the present invention, one would generally contact a cell in need thereof with a lentiviral vector comprising a therapeutic gene. The cell will further be in an organism such as a human in need of the gene therapy. The routes of administration will vary, naturally, with the location and nature of the disease, and include, *e.g.*, intravenous, intrarterial, intradermal, transdermal, intramuscular, intranasal, subcutaneous, percutaneous, intratracheal, intraperitoneal, intratumoral, perfusion and lavage. The cells will also sometimes be isolated from the organisms, exposed to the lentivector *ex vivo*, and reimplanted afterwards.

Injection of lentiviral nucleic acid constructs of the invention may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needleless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

Solutions of the nucleic acids as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intraarterial, intramuscular, subcutaneous, intratumoral and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of

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hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption

delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" or "pharmacologically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic lentiviral vector is delivered to a target cell.

For gene-therapy to discrete, solid, accessible tumors, intratumoral injection, or injection into the tumor vasculature is specifically contemplated. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals. Systemic administration is preferred for conditions such as hematological malignancies.

Continuous administration also may be applied where appropriate. Delivery *via* syringe or catherization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment.

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Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

5   Treatment regimens may vary as well, and often depend on type of disease and location of diseased tissue, and factors such as the health and the age of the patient. The clinician will be best suited to make such decisions based on the known efficacy and toxicity (if any) of the therapeutic formulations based on lentiviral vectors of the present invention.

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The treatments may include various "unit doses." A unit dose is defined as containing a predetermined-quantity of the therapeutic composition comprising a lentiviral vector of the present invention. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit

15   dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of transducing units (T.U.) of lentivector, as defined by tittering the vector on a cell line such as HeLa or 293. Unit doses range from 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} T.U. and higher.

20

G. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor

25   to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

30

Materials and Methodology Employed in Examples 1 through 5

Vector preparation

Production of MLV- and HIV-derived vectors pseudotyped with the vesicular stomatitis virus (VSV) G envelope protein was achieved by transient co-transfection of three plasmids into 293T epithelial cell line as described in Naldini *et al* 1996a. MLV vector particles were produced using the CMV-GagPol plasmid as packaging construct (Scharfmann *et al.*, 1991), and a vector derived from pSLX, which expressed GFP from the CMV promoter. The HIV-derived packaging construct used was pCMVΔR8.91, which encodes the HIV-1 Gag and Pol precursors, as well as the regulatory proteins Tat and Rev (Zufferey *et al.*, 1997). VSV G was expressed from pMD.G.

The HIV vector plasmids were derivatives of the original pHR' backbone (Naldini *et al.*, 1996a), with the following modifications. Self-inactivating vectors were produced from the previously described SIN-18 vector, which contains a deletion in the U3 region of the 3'LTR from nt. −418 to nt. −18, removing all the transcriptionally active sequences (Zufferey *et al.*, 1998). Briefly, the pHR'SIN plasmids (also see maps in FIG. 6, FIG.7, FIG.9 and FIG.9) were generated as follows; a *KpnI-XbaI* fragment containing the polypurine tract and the 3'LTR was excised from a pHR' plasmid and subcloned into the corresponding sites of pUC18. This plasmid was digested completely with *EcoRV* and partially with *PvuII* and self-ligated. A plasmid carrying a 400-nucleotide deletion of U3 was recovered. An *XhoI* linker was inserted into the *EcoRI* site of the deletion plasmid, and an *XhoI-XbaI* fragment was cloned back into the pHR' CMVlacZ plasmid digested with the corresponding enzymes. All other SIN-18 plasmids were obtained by substituting reporter genes (encoding luciferase, GFP and Neo) for lacZ. The pHR' vector plasmids used differed from the plasmids originally described (Naldini *et al.*, 1996) by a *XhoI-KpnI* deletion removing 118 nucleotides from the *Nef* coding sequence upstream of the polypurine tract and a deletion of 1,456 nucleotides of human sequence downstream of the 3' LTR. This human sequence remained from the original cloning of a HXB2 proviral genome. The two deletions did not affect vector titers or transgene expression in dividing 293T cells.

Insertion of the EF1alpha was done by inserting a *ClaI-BamHI* cassette containing a EF1-GFP insert, into *ClaI-BamHI* site of pHR'-GFP-W-SIN (Zufferey et al. 1999)

The maps shown in FIG. 6, FIG.7, FIG.9 and FIG.9 depict the plasmid constructs pHR-CMV-GFP, pHR-EF1-GFP, pHR-EF1-GFP-SIN and pHR-EF1-GFP-W-SIN.

The phosphoglycerate kinase (PGK) promoter corresponded to nucleotides 424 to 930 of the murine PGK gene (Genbank accession number M18735). The EF1α promoter was derived from the pEF-BOS plasmid (Mizushima and Nagata, 1990), and comprised nucleotides 373 to 1561 of the human elongation factor EF1α gene (Genbank accession number: J04617). After transient transfection of the three plasmids by calcium phosphate in 293T cells, the supernatant was harvested, concentrated by 2 rounds of ultracentrifugation, and resuspended in serum-free Cellgro® SCGM medium (BioWhittaker Europe, Verviers, Belgium). Viral stocks were stored at -70° C and titers determined by transduction and flow cytometry analysis of GFP expression in HeLa cells as previously described (Zufferey *et al.*, 1997). Titers were comprised between $5x10^7$ and 10^8 HeLa-transducing units (TU) per ml.

Purification and transduction of CD34$^+$ cells

Cord blood (CB) samples were obtained according to institutional guidelines and CD34$^+$ cells were purified as described (Arrighi *et al.*, 1999). In brief, CB mononuclear cells recovered after Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation were incubated on ice with anti-CD34 M450 Dynabeads as described by the manufacturer. After several washes to eliminate unbound cells, CD34$^+$ cells were recovered from the beads by incubation for 15 minutes at 37°C with the "Detach-a-bead" included in the kit. Cells were immediately washed, and analyzed by flow cytometry. The percentage of purified CD34$^+$ cells was 89 ± 7.0. For transduction, 10^5 cells were seeded in 96-well plates in 100 µl of Cellgro® SCGM medium supplemented with antibiotics (Gibco BRL, Life Technologies LTD, Paisley, Scotland, U.K.), with 10^{-4} M dithiothreitol (Fluka Biochemika, Buchs, Switzerland) and TPO. After overnight incubation, 10^6 (typically), or 10^5 to $5x10^6$ (for dose-response analysis) HeLa-

transducing units (TU) of vector were added per well, and the volume was adjusted to 200 µl with Cellgro® SCGM medium containing TPO. After 24 hours, cells were washed, diluted to 400 µl in IMDM supplemented with 10% of FCS (both from Gibco BRL, Life Technologies LTD), antibiotics, and FLT3-L, TPO and SCF for 3 days. Cells

5   were either directly analyzed for GFP and CD34 expression, or further cultured with the 3 growth factors. For experiments shown in FIG.1, concentrated viral stocks were treated with DNAse I prior to incubation with cells in order to eliminate DNA contaminants that may interfere with further PCR analysis. Briefly, 10^6 TU of vector was incubated for 30' at 37°C in a final volume of 20 µl containing 20 µg/ml Dnase I (DNAse I, Roche

10   Diagnostics, Rotkreuz, Switzerland) and 10 mM MgCl2. Then, 80 µl of Cellgro was added and the resulting 100 µl were added to 100 µl of Cellgro containing10^5 CD34+ cells. Cells were then cultured as described above.

PCR analysis after transduction of CD34$^+$ cells

15   Cells (5×10^5) were lysed in 18 µl of 50 mM Tris.HCl, pH 8.0, 20 mM NaCl,1 mM EDTA, 1% SDS. Proteinase K (2 µl at 10 mg/ml) was added and samples were incubated for 30' at 55°C. After digestion, 200 µl of water was added and samples were boiled for 10' to inactivate proteinase K. PCR amplification was performed using HotStarTaq (Qiagen, GmbH, Hilden, Germany) in a final volume of 50 µl and 2 µl or 0.7 µl of extract

20   with the following cycles: 95°C 15'; 40 cycles 94°C 30", 60°C 1', 72°C 1', plus 10' extension phase at 72°C. Sequences of the PCR primers were: 5' hIL2 gcaactcctgtcttgcattg; 3' hIL2 aatgtgagcatcctggtgag,; 5'GFP gtgagcaagggcgaggagc; 3'GFP cttgatgccgttcttctgcttgt. Conditions were adjusted to observe decrease in final PCR product yield when diluting samples by a factor of 3 (see FIG. 1A).

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Purification and transduction of primary human T cells

Peripheral blood mononuclear cells were purified from buffy coats of healthy donors over a Ficoll-Hypaque gradient. Macrophages were removed by plastic adherence for 2 hrs at 37° C, and the remaining cells were incubated with a cocktail of monoclonal

30   antibodies against HLA-DR, CD25, CD69, CD19, CD16, CD11b and CD14 (Pharmingen). After a 30-min incubation on ice, cells were washed twice and incubated

with magnetic beads (Dynabeads; Dynal, Oslo, Norway) conjugated with goat anti-mouse IgG, at a 1:4 target/bead ratio. Thirty minutes later, bead-bound cells were removed using a magnet. Remaining cells were further purified through two more rounds at an increased target/bead ratio (1:10). This purification protocol typically resulted in a 99.5% pure population of resting T cells, as determined by flow cytometry analysis with antibodies against the activation markers HLA-DR, CD25 and CD69. Cells maintained in RPMI 1640 supplemented with 10% FCS, 5 mM penicillin and streptomycin (GIBCO BRL) and 2 mM glutamine (GIBCO BRL) were activated with phytohemagglutinin (Sigma) at 3 μg/ml for 48 hrs and subsequently cultured in RPMI 1640 containing 10% fetal calf serum and recombinant interleukin-2 (Sigma) at 10 U/ml. 5×10^5 cells were transduced with various lentiviral vectors at a MOI of 5 HeLa-transducing unit per cell in 24-well plates in the presence of PHA, in a final volume of 500 μl. Flow cytometry analysis of GFP expression was performed five days after transduction.

Cytokines

All cytokines were recombinant human material. GM-CSF (Leucomax) from Essex Chimie & Sandoz (Basel, Switzerland) was used at 20 ng/ml, G-CSF (Neupogen) from Roche (Basel, Switzerland) at 10 ng/ml, and erythropoietin (Eprex) from Cilag (Schaffhausen, Switzerland), at 2U/ml. Other cytokines were purchased from Peprotech EC (London, U.K.) and used at the following concentrations: FLT3-L 25 ng/ml, TPO 10 U/ml, SCF 20 ng/ml, interleukin-3 10 ng/ml, TNF 40 ng/ml and IL-4 20 ng/ml.

Antibodies and immunoreactants

Phycoerythrin-conjugated monoclonal antibodies (MAb) were the following: anti-CD34 (mIgG1 clone 8G12) from Becton-Dickinson (Mountain View, CA), anti-glycophorin-A (mIgG1, clone JC 159), anti-CD42b (mIgG2a, clone AN51) an isotypic controls from Dako A/S (Glostrup, Denmark). Biotin-labeled MAbs: Anti-CD14 (mIgG2a, clone UCHM1) from Ancell Corp (Bayport, MN), and anti-CD15 (mIgM cloneDU-HL60-3) from Sigma (St Louis, MO), and isotypic controls from Ancell. Allophycocyanin (APC)-labeled streptavidin was from Pharmingen (San Diego, CA). Anti-CD34 mIgG coated M450 Dynabeads were from Dynal A/S (Oslo, Norway).

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In vitro differentiation

Megakaryocytic differentiation was evaluated after 10 days of expansion culture with FLT3-L, TPO, and SCF. For the other lineages, cells were washed after 9-11 days of expansion culture with FLT3-L, TPO, and SCF, and incubated with EPO, IL-3 and SCF for erythroid differentiation, GM-CSF and SCF for monocytic differentiation, and G-CSF and SCF for granulocytic differentiation. Differentiation into dendritic cells was performed as previously described (Arrighi *et al.*, 1999). Briefly, transduced CD34+ cells were expanded for 14 to 28 days with FLT3-L, TPO, and SCF. Cells were then induced into mature DC by exposure to GM-CSF and IL-4 for 3 days, followed by GM-CSF, IL-4 and TNF for 3 more days. The amplification ratio was comprised between 100 and 1000 depending on the experiment and the cell lineage.

Flow cytometry analysis

Cells were analyzed as described (Arrighi *et al.*, 1999), on a FACScalibur (Becton-Dickinson) with slight modifications. FL-1 was used for GFP, FL-2 for PE-labeled MAbs, FL-3 for identification of living cells with the non-permeant DNA dye 7-amino-actinomycin D (Sigma) (Schmid *et al.*, 1994), and FL-4 for biotinylated MAbs indirectly labeled with streptavidin-APC. Cell suspensions were adjusted to 0.5% paraformaldehyde prior to analysis. Data were analyzed using WINMDI software written by J. Trotter at Scripps Institute (La Jolla, CA) and CellQuest software (Becton-Dickinson).

Example 1

Transduction of Human Hematopoietic Progenitors with MLV Vectors and HIV-Based Lentivectors

Human cord blood CD34$^+$ cells were purified by a single round of positive selection with magnetic beads, resulting in preparations approximately 90% pure. Cells were maintained overnight in serum-free medium supplemented with TPO, and were exposed for 24 hrs to MLV- or HIV-based GFP-expressing vectors pseudotyped with the

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G protein of vesicular stomatitis virus (VSV G), prepared by transient transfection of 293T cells as previously described (Naldini *et al.*, 1996a; Zufferey *et al.*, 1997). The lentiviral packaging system used in these experiments was of a so-called second generation (Zufferey *et al.*, 1997), comprising the HIV-1 *gag, pol, tat* and *rev* genes. The multiplicity of infection (MOI) was 10 HeLa-transducing unit (TU) per CD34$^+$ cell. After transduction, cells were incubated for another three days in medium containing FCS, FLT3-L, TPO and SCF, and analyzed by flow cytometry for GFP (FIG.1) and CD34 expression and lysed for PCR analysis. The relative potencies of three internal transcription units were compared within the context of lentivectors: the cytomegalovirus immediate early (CMV), the phosphoglycerate kinase (PGK) and the alpha chain of elongation factor 1α (EF1α) promoters.

As shown in FIG. 1A, a sharp subpopulation of GFP-positive hematopoietic progenitors could only be seen when cells were transduced with HIV vectors containing the PGK or the EF1α promoters. Cells transduced with MLV vector or HIV vector containing the CMV promoter displayed only a small percentage of GFP-positive cells, together with a high heterogeneity in GFP expression. A side-by-side comparison of GFP expression after transduction of HeLa and CD34$^+$ cells revealed that the promoters examined behaved quite differently in these two cell types. The PGK promoter was weak in HeLa cells (overlap between GFP$^+$ and GFP$^-$ cells) and strong in HPCs. The EF1α promoter was intermediate in HeLa cells and very potent in HPCs, with a mean value typically 100 times higher in transduced than in control HPCs. The CMV promoter, whether in an MLV or an HIV vector, was equally strong in HeLa cells, contrasting with its low activity in transduced HPCs. A PCR-based quantification performed at the time of the flow cytometry analysis (FIG. 1B) indicated that the low expression from the MLV-CMV vector in HPCs was due at least in part to poor gene transfer in these cells, a consequence of the limited stimulation of CD34$^+$ cells during transduction. In contrast, the low expression in HPCs exposed to the HIV-CMV vector (FIG. 1A) could not be explained by a low frequency of transduction, since equivalent amounts of transgene DNA were present in cells transduced with all three HIV vectors (FIG. 1B). This indicates that the CMV promoter does not govern efficient expression in hematopoietic

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progenitors, a finding in accordance with previous reports (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000).

The inventors also ascertained that GFP expression originated from integrated proviruses. For this, a PCR analysis was performed following 3 weeks of expansion of the transduced CD34$^+$ cells. During this time period, the cell numbers increased by a factor of 900 to 1800, depending on conditions and experiments. In the particular experiment illustrated here (FIG. 1B), the percentage of cells expressing GFP from the PGK promoter decreased by a factor of approximately 2 after expansion and differentiation into erythroid or monocytoid cells, which paralleled a slight decrease in GFP DNA copy number. This confirms that the bulk of transgene DNA remains in the cells after massive expansion, excluding its presence as unintegrated forms. This also correlates the previous demonstration that long term transgene expression cannot be detected after transduction with integrase-deficient HIV-vectors (Naldini *et al.*, 1996b).

Thus, the EF1α promoter induces high transgene expression in lentivector-transduced CD34$^+$ cells

Example 2
Transduction of Human CD34$^+$ Cells as a Function of Increasing Vector Concentration

The high levels of GFP expression induced by the EF1α-containing HIV- derived vector allowed for a reliable determination of gene transfer efficiency, because even low numbers of transduced cells were easily detected. This vector was thus used to evaluate the influence of the MOI on the transduction efficiency of CD34$^+$ cell (FIG. 2). Although the percentage of GFP$^+$ cells initially increased as a direct function of the MOI, the curve flattened starting at a MOI of 5, reaching a maximum of 25 % GFP$^+$ cells (±5% according to experiments) at MOIs 20 and above. In the absence of optimal cytokine-induced proliferation, only a fraction of human CD34$^+$ cells is thus permissive to lentivector-mediated transduction, as previously suggested (Sutton *et al.*, 1999).

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Example 3

Transgene Expression in Hematopoietic Lineages after Transduction of CD34$^+$ Cells with HIV Vectors Containing PGK and EF1α Promoters

An important issue for the genetic treatment of a variety of lympho-hematological disorders will be the levels of expression of the putatively therapeutic transgene in the appropriate subset of differentiated cells. In that respect, recent reports using HIV-based vectors with an internal expression cassette driven by the CMV promoter have documented the incapacity of this transcriptional element to induce strong transgene expression in precursors as well as mature blood cells (Miyoshi *et al.*, 1999; Case *et al.*, 1999). To identify more suitable alternatives, hematopoietic progenitors transduced with HIV-based vectors containing the PGK or the EF1α promoters were differentiated *in vitro* to compare the relative potency of these vectors in various hematopoietic lineages. For this, transduced CD34$^+$ cells were first expanded for 7-14 days in FLT3-L/TPO/SCF and then incubated in various differentiating media for an additional 5-to-10-day period, allowing the generation of erythroid cells, granulocytes and monocytes. Megakaryocytes were induced in FLT3-L/TPO/SCF exclusively. For dendritic cells (DC), transduced CD34$^+$ cells were expanded for 14 to 28 days with FLT3-L/TPO/SCF. Cells were then induced into mature DC by exposure to GM-CSF/IL-4 for 3 days, followed by exposure to GM-CSF/IL-4/TNF for 3 more days.

The differentiated cells were then analyzed by flow cytometry to determine the percentage of lineage-specific marker$^+$ / GFP$^+$ cells, as well as the relative levels of GFP expression in the differentiated populations (FIG. 3). Transgene expression was high in all lineages examined after transduction of precursors with the EF1α-containing lentiviral vector, with a signal-to-noise ratio (mean of fluorescence intensity of GFP$^+$ cells divided by mean of fluorescence intensity of GFP$^-$ cells) comprised between 150 and 200 depending on the cell type. The PGK promoter induced lower and more variable levels of GFP expression in differentiated cells. Expression of GFP under the control of the PGK promoter was highest in DCs where it was only 3.5 less potent than that driven from

the EF1α promoter, and worst in erythroid cells (glycophorin$^+$), where it was 11 fold weaker than its EF1α counterpart. However, even in this case, the signal-to-noise ratio was still high enough to permit a clear discrimination between GFP$^+$ and GFP$^-$ cells, allowing for the reliable analysis of transgene expression.

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Taken together, these data indicate that the transduction of human CD34+ cells with HIV-based vectors containing internal promoters derived from the EF1α and to a lesser extent the PGK gene results in high levels of transgene expression in several hematopoietic lineages.

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Example 4

EF1α Promoter-Based Lentiviral Vectors Induce
High Levels of Transgene Expression in Primary T Lymphocytes

15   The system used in these studies did not allow for the easy differentiation of CD34$^+$ precursors into cells of the lymphocytic lineage. Therefore, to compare the relative value of CMV-, PGK- and EF1α-containing HIV-derived vectors in these cells, mature T-lymphocytes purified from the peripheral blood were directly used as transduction targets (FIG. 4). The results revealed the same hierarchy as observed in

20   HPCs and in the various other blood cell lineages, with the EF1α promoter inducing levels of GFP expression significantly higher than those yielded by the PGK promoter, whereas cells transduced with CMV-based vectors exhibited levels of GFP expression that were incompatible with proper detection by flow cytometry. Experiments using human primary B cells show that the CMV promoter is very active in these cells. This

25   indicates that the poor activity of the CMV promoter is not universal in human hematopoietic cells.

Example 5

Influence of SIN Design and WPRE on Transgene Expression

The use of self-inactivating (SIN) 3'LTR U3-deleted HIV vectors increases the
biosafety of this system and avoids a possible interference between the viral LTR and the
vector's internal promoter (Zufferey *et al.*, 1998). EF1α- and PGK-containing HIV-
derived SIN vectors were therefore tested for their ability to induce high levels of
transgene expression in hematopoietic precursors (FIG. 5). The SIN design was
accompanied by a dramatic decrease (6 fold) in GFP expression within the context of the
PGK vector, whereas it instead had a slightly yet reproducibly positive effect when
introduced in the EF1α vector. Inserting the sequence for the woodchuck hepatitis virus
posttranscriptional regulatory element (WPRE) (Donello *et al.*, 1998) upstream of the 3'
LTR has been shown to promote transgene expression from HIV-derived vectors in some
targets (Zufferey *et al.*, 1999). It indeed stimulated GFP production from the PGK
promoter by a factor of 2.15 in hematopoietic precursors. In contrast, it exerted a
negative effect on expression from the highly active EF1α promoter, with a decrease by a
factor of 1.85. Taken together, these indicate that the strong EF1α promoter is less
sensitive than the PGK promoter to a self-inactivating configuration, making it a better
candidate for gene expression in hematopoietic cells.

All of the compositions and/or methods disclosed and claimed herein can be made
and executed without undue experimentation in light of the present disclosure. While the
compositions and methods of this invention have been described in terms of preferred
embodiments, it will be apparent to those of skill in the art that variations may be applied
to the compositions and/or methods and in the steps or in the sequence of steps of the
method described herein without departing from the concept, spirit and scope of the
invention. More specifically, it will be apparent that certain agents which are both
chemically and physiologically related may be substituted for the agents described herein
while the same or similar results would be achieved. All such similar substitutes and
modifications apparent to those skilled in the art are deemed to be within the spirit, scope
and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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